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Viability of unstimulated lymphocytes exposed to extremely low frequency electromagnetic fields is dependent on intensity

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Abstract

The cell viability and DNA damage in unstimulated sheep primary lymphocytes subjected to different extremely low electromagnetic field intensities (5, 50 and 100 μT; 50 Hz) were studied with special emphasis on apoptosis. Sheep primary lymphocytes cultured in RPMI, supplemented with 10% FBS in the absence of mitogens, were exposed till 16 h. The cell viability assessment by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay showed a dose dependent enhancement of viability at 16 h. Further, quantitative DNA laddering and flow cytometric analysis showed a significant decrease in apoptosis of the cells subjected to 100 ($p<0.01$) and 50 μT ($p<0.05$) for 16 h as compared to control group. There was a statistically significant decrease ($p<0.01$) in the specific activity of caspase 9 at 100 μT in cells exposed for 16 h. However, no enhancement of DNA damage was observed at 5, 50 and 100 μT as evidenced by comet assay. Comet assay also confirmed the decreased cell death of exposed cells (100 μT). Experimental data suggests decreased apoptosis at 100 μT (50 Hz), possibly by the suppression of caspase 9 activity leading to the enhanced cell viability.

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Introduction

The biological effects caused by Extremely Low Frequency Electromagnetic fields (ELF-EMF) have been undergoing serious debate since the last two decades. Several studies have been conducted to asses the effect of ELF-EMF, during this period using different biological models - animal model [1] cellular model [2], developing embryos [3] and plants [4] so as to explain the possible mechanism [5]. The first pioneering epidemiological study by Werthiemer and Leeper [6] put forward a relationship between EMF exposure and childhood leukemia. The present study on cellular models was mainly aimed to obtain a relationship between ELF-EMF and biological effects.

However, the available biological data on the oncogenic potential of ELF-EMF are unable to provide a cause-effect relationship between exposure conditions and induction of tumors. In this context, the question of possible genotoxic effects is particularly relevant. It has been reported that EMF leads to chromosomal breaks, changes in direction, formation and functionality of mitotic spindle and may have genotoxic effects [7] in various cells. There are also other contradictory studies,

which shows that EMF may not have any influence on frequencies of sister chromatid exchanges [8, 9], cell cycle progression or chromosomal breakage [10] in lymphocytes. There are several reports indicating possible induction of mutation, following EMF exposure. Single strand DNA breaks have been reported in Molt 4 T-lymphoblastoid cells [11]. A dose dependent increase in DNA single strand breaks was observed in human leukocytes exposed to 50 Hz magnetic fields [12]. The latest published data provided evidence suggesting that ELF-EMF interfere with the DNA repair process leading to accumulation of damaged DNA in cells [13]. It was also stated by Philip et al., 1996 [11], that exposure of Molt 4 T- lymphoblastoid cells to a 100 μT, 60 Hz sinusoidal magnetic fields leads to decreased activity of the DNA repair enzyme, poly (ADP ribose) polymerase, and increased DNA fragmentation in the presence of the DNA damaging agent, etoposide. However, there are negative reports in the literature [14, 15, 16] that indicates no detrimental effect of ELF-EMF on cellular DNA. The studies conducted *in vitro* and on cell free DNA mismatch repair revealed that the core processes related to the transmission of genetic information (DNA polymerase activity, transcription and repair) are stable under ELF-EMF (60 Hz, at 0.25 to 0.5 T) [17]. Similarly, environmental level magnetic fields (10, 50 and 100 μT) had no effect on *in vitro* transcription in a cell free system [18].

Many other studies have also shown an increase in proliferation after exposure to EMF in human fibroblasts [19] and lymphocytes [9, 20, 21, 22]. The exposure of 60 Hz magnetic fields (1.0, 1.5 and 2.0 mT) on human lymphocytes was found to modify the cell proliferation and had no influence on the frequency of sister chromatid exchanges [23]. Similarly, ELF-EMF was found to induce partial blockage for the differentiation of Friend erythroleukemia cells, which results in a larger population of cells remaining undifferentiated in the proliferative state suggesting that ELF-EMF may act as a tumor promoter [24, 25].

Earlier studies have reported an increase in apoptosis in human myelogenous leukemic cell lines, HL-60 and ML-1 [2, 26], in thymocytes and macrophages of mice exposed to long term ELF-EMF [27, 28]. In macrophages of mice, the increase in apoptosis was accompanied by increased intracellular calcium levels [27]. Similarly, Ca^{2+} movement was induced in U-373 MG cells by ELF-EMF (50 Hz, 3mT), however there was neither cell proliferation nor cell death [29]. Thymocytes from mice subjected to a long term 0.4 to 1.0 mT, 60 Hz, showed an abnormal response to dexamethazone induced apoptotic stimuli [28]. In contrast, the static and ELF magnetic fields possessed an anticancer activity [30]. Similar effect was observed in three different human tumor cell lines [HTB63 (melanoma), HTB77 IP3 (ovarian carcinoma) and CCL86 (lymphoma)] subjected to prolonged exposure of static magnetic field [31]. The continuous exposure to 1.2 μT, sinusoidal 60 Hz magnetic field was found to influence chemically induced proliferation control processes in breast cancer cells (MCF-7) in vitro [32].

In view of the mutually contradictory reports on the oncogenic or proliferative effect of ELF- EMF on cellular models, the effect of ELF-EMF on cell viability and DNA damage in primary lymphocytes (non-transformed) subjected to ELF-EMF with special emphasis on apoptosis was investigated. The magnetic field dosage (5, 50 and 100 μT) was selected based on the national survey of magnetic fields conducted by Central Power Research Institute, Ultra High Voltage Research Laboratory, Hyderabad, India (unpublished data; CPRI report No.11.1.11 2000).

Results

Influence of magnetic fields on viability of lymphocytes

The lymphocyte viability based on MTT assay at different magnetic fields is given in Figure 1. It was observed that upon exposure to 5, 50 and 100 μT for 16 h, there was a dose dependent enhancement in the viability of lymphocytes with reference to control group. Statistically significant ($p<0.05$) increase in the viability of lymphocytes subjected to 100 μT for 16 h as compared to control group was observed (Figure 1).

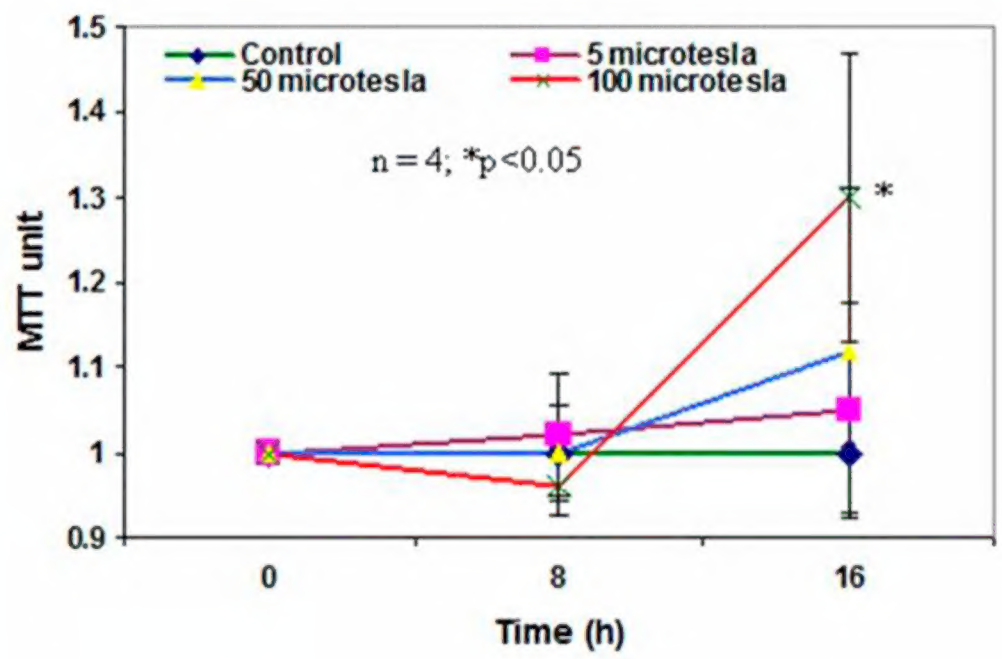


Figure 1. Effect of magnetic field (5, 50 and 100 μT) on lymphocyte viability.

DNA damage analysis by comet assay

The percent lymphocytes, categorized as "no tail", "nominal tail" and "longer tail" along with positive control (Figure 2a) based on comet assay are given in Figure 2b. There was a statistically significant ($p<0.01$) decrease in the percent of "nominal tail" lymphocytes subjected to 100 μT for 16 h (Figure 2b). There was also a gradual increase in the percent of "no tail" lymphocytes subjected to 5, 50 and 100 μT for 16 h in comparison with the control group.

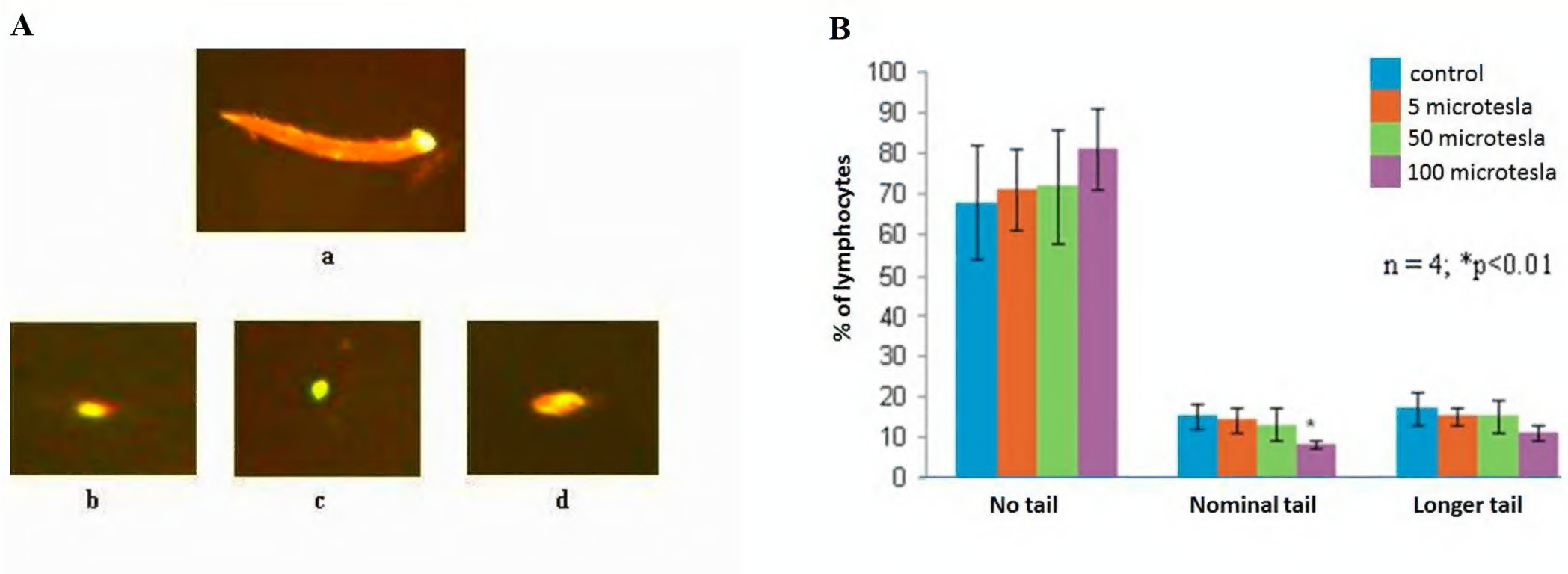


Figure 2. A. Categorization of cells based on comet tail length [a: positive control (H_2O_2 treated); b: nominal tail; c: no tail; d: longer tail]. **B.** Depending on the tail length, the cells were categorized into nominal and long tail by scoring 100 cells. Cells, without tail were scored separately.

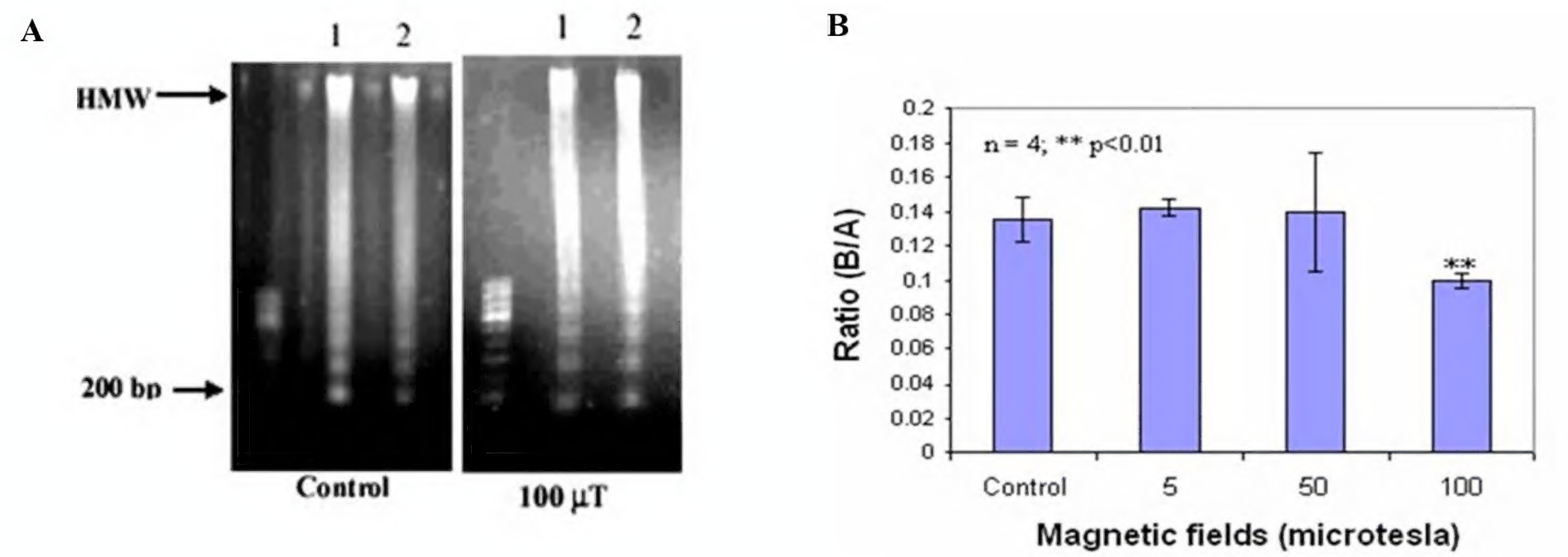


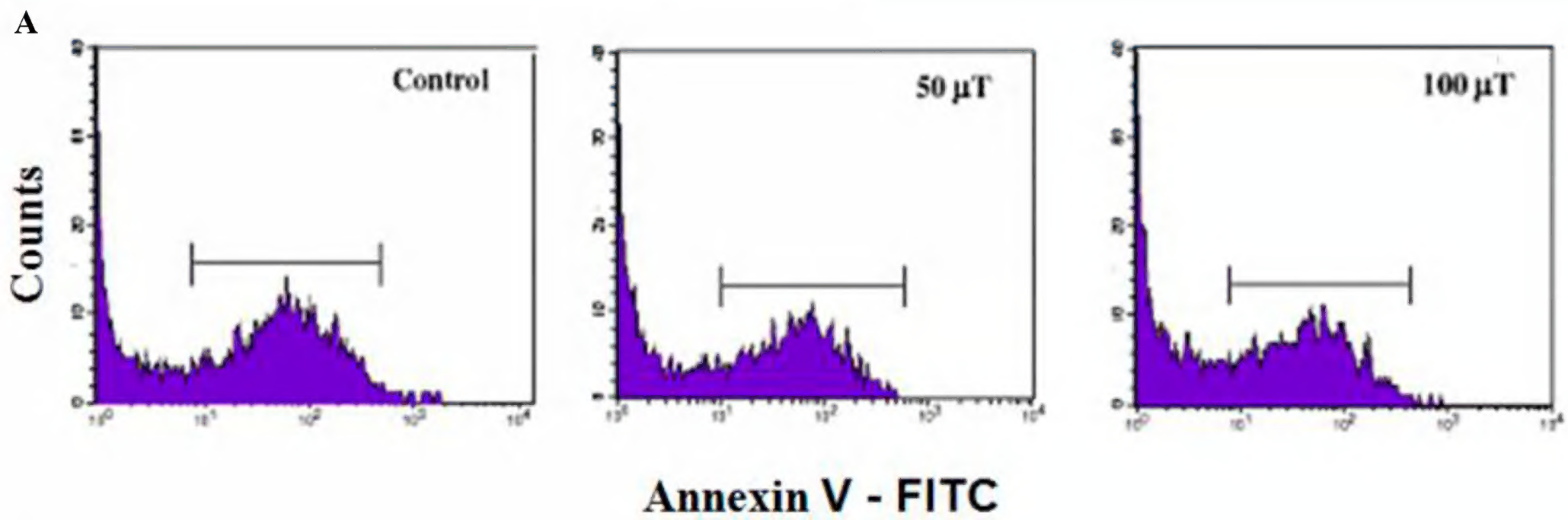
Figure 3. A. DNA laddering analysis (Lane 1 and 2 represents samples in duplicate) showing distinct band of High Molecular Weight (HMW) DNA from viable cells and 200 bp DNA band from dead cells. **B.** Effect of magnetic fields (5, 50 and 100 µT) on % Fluorescent Intensity (FI) ratio of 'B' (200 bp DNA) / 'A' (HMW DNA). A statistically significant ($p<0.01$) decrease in the ratio in cells subjected to 100 µT for 16 h, compared to control group was seen.

Influence of magnetic fields on cell death (DNA laddering analysis)

DNA laddering analysis of lymphocytes showed distinct bands of high molecular weight (HMW) and 200 bp DNA (Figure 3a). The laddering pattern was found to be similar in both control and exposed groups of cells. However, the comparison of percent fluorescent intensity ratio {[FI of 200 bp DNA (B) / FI of HMW DNA (A)] x 100} revealed that there was a statistically significant ($p<0.01$) decrease in the ratio in cells subjected to 100 µT for 16 h (Figure 3b), whereas, the cells subjected to 5 and 50 µT showed no effect in the ratio as compared to control group.

Influence of magnetic fields on apoptosis of lymphocytes (Annexin V staining)

Annexin V stained lymphocytes, subjected to different magnetic fields (5, 50 and 100 µT) for 16 h are represented by histogram obtained by Fluorescence activated cell sorting (FACS) analysis (Figure 4a). The histogram indicates the reduction of peak size corresponding to Annexin V- FITC bound cells in magnetic field exposed groups (50 and 100 µT) as compared to the control group. The quantitative analysis of percent of Annexin V - FITC bound cells (apoptotic cells) showed a statistically significant ($p<0.05$ and $p<0.01$) reduction of apoptotic cells at 50 and 100 µT



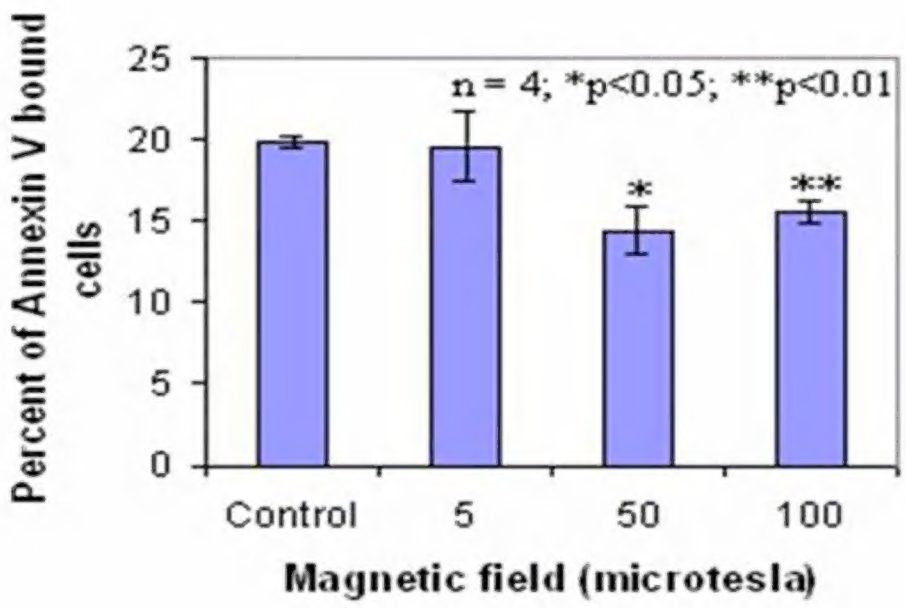


Figure 4. A. Flow Cytometric analysis of apoptosis. **B.** Flow cytometric quantitation of apoptotic cells was carried out using Annexin V-Biotin Apoptosis Detection kit, which uses Annexin V-Biotin conjugate for detection of cell surface changes that occur early in the apoptotic process

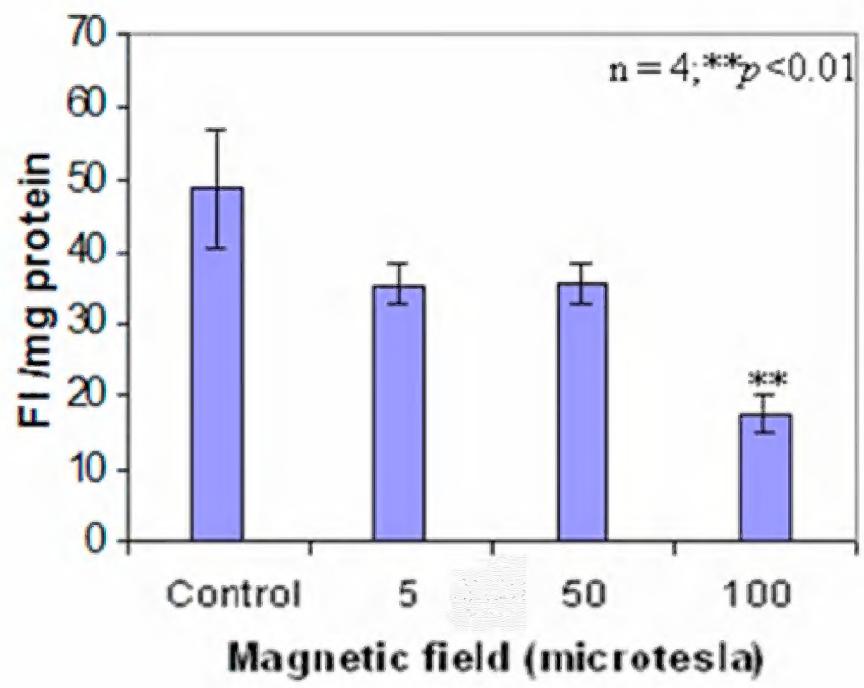


Figure 5. Spectrofluorometric analysis of caspase 9 activity. The caspase 9 activity was expressed as FI/mg protein by estimating the protein content in the cell lysate using detergent friendly BCA method.

respectively as compared to control group. However, there were no changes in the percent of apoptotic cells at 5 µT (Figure 4b). This clearly gives a quantitative picture of reduction of apoptosis in primary lymphocytes subjected to EMF stress in the absence of any phytostimulator.

Influence of magnetic fields on apoptosis of lymphocytes (caspase 9 activity)

The specific activity of caspase 9 in lymphocytes exposed to different magnetic field intensities (5, 50 and 100 µT) for 16 h are given in Figure 5. It is evident that there was a statistically significant decrease ($p<0.01$) in

specific activity of caspase 9 in lymphocytes subjected to 100 μT as compared to control group. The specific activity of caspase 9 of lymphocytes subjected to 5 and 50 μT did not show any change as compared to control group.

Discussion

There are numerous reports, which indicate that power frequency (50 Hz) magnetic fields induce responses at cellular level, particularly in peripheral lymphocytes. Peripheral lymphocytes have been the cellular model of choice because they are well characterized cells as far as culture conditions and growth factor requirements are concerned. Lymphocytes are usually obtained as resting cells from peripheral blood, but can be induced to enter the cell cycle using mitogens, thus mimicking a naturally occurring activation process [22]. The mitogenic response in lymphocytes was reported to be stimulated in the presence of ELF-EMF *in vitro* [33, 34, 21]. However, there are other reports, which indicate that the mitogenic response in lymphocytes was inhibited [35, 36] or not influenced [10]. There are very few reports in contemporary literature on the effect of ELF-EMF on the unstimulated cells [37, 38]. Vincze et al., 2005 [39] suggested based on theoretical calculations that external low frequency electromagnetic fields (LFEMF) can induce a distribution of field strengths along the cell membrane. This leads to thermal gradients (currents) from the extracellular matrix towards the inside of the cell. This thermal current also carries ions through, leading to thermo-diffusion and thus creating a zero mode electric current, which in turn induces a zero mode electric field in the cell membrane. Therefore, even small fields with zero mode components could elicit biological effects. Further, it has been shown in the previous investigations, that ELF-MF exposure affected the conformation of chromatin in *E. coli* and human lymphocytes, when used as test systems. ELF-MF, under specific conditions of exposure, acted as a non-toxic but cell-growth stimulating agent, when conformation of chromatin was studied by the method of anomalous viscosity time dependencies (AVTD) method [40, 41]. The present experimental studies were focused mainly to address the biological effects of ELF-EMF (50 Hz) generated during the course of high voltage power transmission. Hence, the study was conducted at very low dosage of magnetic fields (5, 50 and 100 μT; 50 Hz).

It was earlier established that lymphocyte cultures should be exposed for a minimum period of 6 h [22] because all processes related to cell proliferation such as RNA synthesis, gene expression, and DNA synthesis occur within 6 h from the binding of the mitogen to the cell membrane. In the present study the lymphocytes have been exposed to magnetic fields for a period of 16 h in the absence of mitogens and a statistically significant increase ($p<0.05$) in the cell viability was observed at 100 μT. Cells subjected to 5 and 50 μT magnetic fields also showed a trend in increased cell viability, even though it was statistically not significant (Figure 1). This observation indicates that EMF can enhance the viability of lymphocytes in the absence of mitogens also. As a supporting evidence, comet assay showed a gradual increase of percent of cells with "no tail" (viable cells) at 5, 50 and 100 μT, even though, the variation was statistically not significant. It was quite interesting to note that there was a statistically significant decrease ($p<0.01$) in the percent of lymphocytes having "nominal tail" at 100 μT, subjected for 16 h (Figure 2b). This indicates that the percent of cells that undergoes natural death (possibly by apoptosis) was found to be decreased under 100 μT. Since there were no mitogens in the medium the chance of cell proliferation under this condition is very low. In this context, the increase in the cell viability at 100 μT assessed by MTT assay may be mainly due to the enhancement of life of lymphocytes by suppressing the natural death. The same effect might be the reason for the co-stimulatory activity of EMF on lymphocytes in presence of mitogens [22]. The comet assay analysis also indicates that there are no chances of DNA damage due to magnetic field exposure up to 100 μT. This observation is supported by the generally accepted fact that EMF has no direct clastogenic effect on cellular DNA [42]. However, there are several reports indicating possible mutation induction following EMF exposure. Single strand DNA breaks have been reported [11]. A dose dependent increase in DNA single strand breaks was observed in human leukocytes exposed to 50 Hz magnetic fields [12]. It was also stated [11] that exposure of Molt 4 T- lymphoblastoid cells to a 100 μT, 60 Hz sinusoidal magnetic fields leads to decreased activity of the DNA repair enzyme, poly (ADP-ribose) polymerase, and increased DNA fragmentation in the presence of the DNA damaging agent, etoposide. Possibly, these alterations in cellular DNA under ELF-EMF may be due to higher dosages of magnetic fields than the one that exists in the normal routine life, in the vicinity of biological system

Since MTT and comet assay strongly suggests the possibility of repression of cell death and there by gradual dose dependent enhancement of viability at 5, 50 and 100 μT, the present study was mainly focused on apoptosis of lymphocytes under EMF stress. The cellular responses to stress can range from adaptive response to cell death. One of the classical adaptive responses involves the induction or activation of highly conserved proteins called stress or heat shock proteins (HSPs). The major HSPs of mammalian cells include proteins of 110, 90, 70, 60, 40 and 27 kDa. Some HSP members are

constitutively expressed whereas others are expressed only after a period of stress (e.g., rise in temperature, exposure to radiation, viral infection etc.). Induction of these proteins in response to stress confers resistance to subsequent stress (thermo tolerance). This resistance is due to the inhibition of apoptosis [42].

The studies conducted on human keratinocytes subjected to 100 μT showed that power line electromagnetic fields cannot induce changes in phosphorylation, localization or expression of the HSP 27 [43, 44]. However, the cultures of human endothelial cell lines exposed to mobile phone radiation (RF-EMF) showed an increased expression and phosphorylation of HSP 27. Phosphorylated HSP27 has been shown to inhibit apoptosis by forming complex with apoptosome (complex of Apaf-1 protein, pro-caspase 9 and cytochrome C), or some of its components, and preventing proteolytic activation of pro-caspase 9 into active form of caspase 9. This in turn prevents activation of pro-caspase 3 which is activated by caspase 9, leading to the inhibition of apoptosis [45, 46].Apoptosis is a highly regulated form of cell death. It is a fundamental physiological process crucial to maintaining homeostasis in multicellular organisms acting as a counterbalance to cell division. All animal cells programmed to undergo apoptosis when they cease to function, are no longer needed or are damaged. Inappropriate apoptosis occurs in certain degenerative diseases, while a reduced level of apoptosis is frequently associated with the development of cancer [47].One of the classic features of apoptosis is the cleavage of genomic DNA into oligo-nucleosomal fragments represented by multiples of 180 - 200 bp. The DNA band corresponding to 200 bp represents the dead cell fraction whereas high molecular weight fraction present on the top of the gel represents live population of cells [48]. The present study showed a statistically significant ($p<0.01$) reduction in the intensity of 200 bp DNA band in lymphocytes exposed to 100 μT as compared to control group (Figure 3a), whereas, the cells subjected to 5 and 50 μT remained unaffected. This indicates a reduction in apoptosis in lymphocytes exposed to 100 μT for 16 h. Further, this observation supports the data obtained by MTT assay and comet assay since a reduction in the apoptosis under EMF stress in a fixed population of cells will result in enhanced viability as compared to the control group.

Since DNA fragmentation is not a definitive test for apoptosis the observation was further subjected to clarification by conducting the flow cytometric analysis of Annexin V staining of apoptotic cells. Annexin V is a protein that preferentially binds phosphtidyl serine (PS) in a calcium dependent manner. In normal, non-apoptotic cells, PS is segregated to the inner leaflet of the plasma membrane. During early stages of apoptosis, this asymmetry collapses and PS becomes exposed on the outer surface of the cells [48]. The primary lymphocytes subjected to 50 and 100 μT for 16 h showed a statistically significant reduction ($p<0.05$ and $p<0.01$ respectively) in Annexin V bound cell population as compared to control group in a dose dependent manner (Figure 4a and 4b). However, there was no change in lymphocytes subjected to 5 μT. This provides a clear quantitative picture of reduction of apoptosis in primary lymphocytes subjected to EMF stress in the absence of any phytostimulator.

Apoptosis is characterized by cell shrinkage, nuclear condensation and oligonucleosomal DNA fragmentation. The biochemical basis for these morphological features of apoptosis can also be the action of a family of proteases called caspases. Mitochondria plays a key role in activating caspases by releasing cytochrome C into cytosol where it binds to Apaf-1, facilitating pro-caspase 9 processing, followed by caspase 9 mediated activation of pro-caspase 3 [49]. Active caspase 3 promotes the manifestation of some of the more classical features of apoptosis. Caspase 9 is one of the stress induced caspases involved in the intrinsic mechanism of apoptotic cell death [50]. In this context, the above observation was further subjected to biochemical investigations by assaying the activity of caspase 9 in primary lymphocytic cell lysate after subjecting to different magnetic field intensities (5, 50 and 100 μT) for 16 h. The study showed a statistically significant reduction ($p<0.01$) in the specific activity of caspase 9 in cells exposed to 100 μT as compared to the control group (Figure 5). Similarly, there was a minor (non-significant) reduction in the caspase 9 specific activity in lymphocytes subjected to 5 and 50 μT.

The reduction in the caspase 9 specific activities under EMF stress further enriches the above observed fact that EMF inhibits apoptosis in primary lymphocytes. The reduction of apoptosis observed in the present study might be a possible reason for the earlier observation where, the proliferation of human peripheral lymphocytes subjected to EMF (50 Hz) was found to be stimulated in the presence of mitogens [33]. Since the molecular mechanism involved in the different type of cellular responses under the influence of ELF-EMF is not yet clear it is difficult to explain the exact mechanism by which apoptosis is regulated under ELF-EMF in the present context. However, the present investigation suggests that the reduction in apoptosis is possibly achieved by inhibiting the activation of caspase 9 by ELF-EMF. The existing literature strongly supports this hypothesis because i) Caspase 9 is one of the stress induced caspases [50] ii) Caspase 9 activity is regulated by phosphorylation. The pro-caspase 9 becomes inhibited upon phosphorylation at Ser 196. The phosphorylation of pro-caspase 9 is mediated by PI3 Kinase/ Akt pathway [51]. Possibly, the biophysical changes of cell membrane in the presence of 50 Hz EMF [52] might increase the

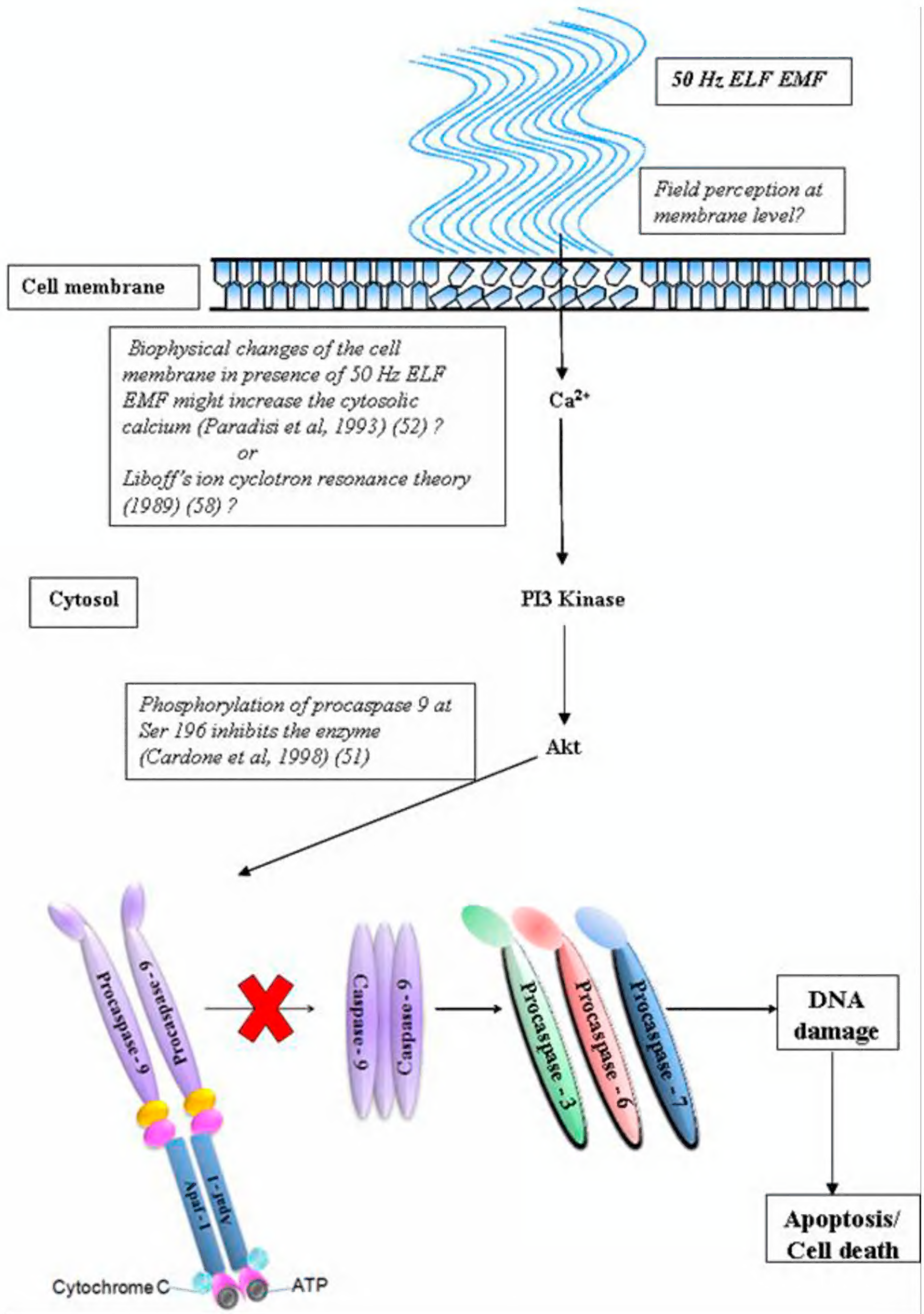


Figure 6. Proposed hypothetical mechanism of inhibition of apoptosis by repression of caspase 9 in primary lymphocytes exposed to 50 Hz ELF-EMF based on the present experimental investigation and contemporary literature [51, 52, 58]. The biophysical changes of cell membrane in presence of 50 Hz EMF might increase the cytosolic Ca^{2+} concentration leading to the activation of PI3 kinase leading to the repression of apoptotic pathway initiated by pro-caspase 9.

cytosolic Ca^{2+} concentration [53, 54, 55] leading to the activation of PI3 kinase which in turn leads to the repression of apoptotic pathway initiated by pro-caspase 9 as shown in Figure 6. Basically, PI3K/Akt pathways function to promote cellular survival following stress. The present hypothesis is further supported by a recent study on human keratinocytes subjected to UV irradiation, which resulted in the inhibition of caspase 9 by PI3K/Akt pathway [56]. Similarly, exposure of B-lineage lymphoid cells to ELF-EMF stimulated the protein tyrosine kinases (Lyn kinase), that plays pivotal and myriad roles in initiation of signal cascades that affect proliferation and survival of B-lineage lymphoid cells [57]. A possible mechanism of inhibition of apoptosis by repression of caspase 9 in primary lymphocytes exposed to 100 μT (50 Hz) ELF-EMF is given in Figure 6.

In summary, the above experimental investigation indicates a reduction in the apoptosis in primary lymphocytes subjected to 100 μT (50 Hz) for 16 h in the absence of mitogens, thereby enhances the viability of the cells. Possibly, the repression of apoptosis in primary lymphocytes is achieved by inhibiting caspases 9, one of the important stress induced initiator caspases. In addition, ELF-EMF (5, 50 and 100 μT; 50 Hz) could not invoke any DNA damage in lymphocytes. It is pertinent to infer that exposure to magnetic fields induces quite a variety of biological effects. Different waveform magnetic field sources results in varied biological effects on living systems, as their frequency spectrums are diverse [39, 58, 59].

Materials and Methods

Materials

Trypan blue, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based in vitro toxicology assay kit and Histopaque-1077 were procured from Sigma (St.Louis, USA). The assay kits for Single cell gel Electrophoresis (comet assay), caspase 9 activity, Annexin V staining were procured from R&D Systems, Inc.(Minniapolis, USA). RPMI 1640, Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from Hyclone (Utah, USA). All other reagents were of analytical grade.

Magnetic field exposure facility

A square-shaped Helmholtz coil, producing ELF-EMFs (50 Hz) proportional to the electric current in the coil, was wound over a Teflon frame of dimension 35 x 35 x 20 cm (l x b x h) suitable for placement inside an air jacketed CO_2 incubator (Binder, Tuttlingen, Germany) as shown in Figure 7. Electric current through the coil was regulated as explained below.

Figure 7. Magnetic field exposure facility fabricated using Teflon frame wound with Helmholtz coil housed inside the CO_2 incubator for studies on cellular models.

Since the magnetic field exposure is a continuous process, an uninterrupted power supply (UPS) was used in order to avoid the supply variation. The output voltage variation of the UPS was limited to less than 1% ensuring stable magnetic field generation. Supply voltage can also produce electric field inside the incubator. If the supply voltage is higher, electric fields produced will also be higher, which is not desirable while studying the biological effects of magnetic fields. To reduce the presence of electric field in the exposure set up, the 230 V supply voltage was stepped down to 15V using a step down transformer. The resistance/impedance of the coil being constant at fixed frequencies, the current control was achieved through a variable resistance connected in series with the coil. Heating effect of the coil was reduced by selecting the conductor of higher diameter suitable for carrying the necessary current. The current source (step down transformer, rheostat etc.) was shielded by means of mild steel sheet enclosures.

The calibration of the exposure facility was carried out to confirm uniform distribution of the magnetic field intensity where the culture flasks would be kept [3].

Isolation of lymphocytes and culturing

Peripheral blood was drawn from the jugular vein of healthy male sheep (1 year old), by using heparinized vacutainers. Lymphocytes were separated by Histopaque-1077 density gradient [60]. The cells were re-suspended in RPMI and an aliquot of 0.1 mL cell suspension mixed with 0.1 mL of 0.4% trypan blue and was applied to the edge of the cover slip of hemocytometer. The viable lymphocytes (unstained) were viewed under 40× using a light microscope and were counted in all the four large corner squares. The cells were cultured (1×10^6 cells/mL) in RPMI 1640 medium (Hyclone) supplemented with 10% FBSm (Hyclone) and 1% penicillin/streptomycin (Hyclone) in

the absence of any phytostimulator. Cultures were divided into two groups:

a) Group I: Control (n = 4, independent trials)

b) Group II: ELF-EMF - exposed (n = 4, independent trials).

The control group was placed in regular CO_2 incubator (Binder, Tuttlingen, Germany) and the experimental group was exposed to varying magnetic field intensities (5 ± 0.25 μT, 50 ± 2.5 μT and 100 ± 5 μT) in the specially designed set up as detailed above. The temperature (37°C) and CO_2 (5%) concentrations were monitored continuously in control and ELF-EMF exposed group.

Isolation of lymphocytes and culturing Cell viability by MTT assay

The cell viability was monitored by the MTT assay. This assay measures the viable cells based on its mitochondrial dehydrogenase activity by which MTT will be reduced to formazan [61]. MTT was added to cells (4×10^6) cultured in 96-well plates (in triplicates) at three time points (0 h, 8 h and 16 h). The cultures were incubated for 5 h at 37°C and analyzed in an ELISA reader at 560 nm after solubilization of formazan crystals. The reduced formazan crystals were dissolved in MTT solubilization solution containing 10% Triton X-100 and 0.1 N HCl in anhydrous isopropanol. The number of MTT units in culture samples subjected to EMF was calculated as the ratio (Abs. sample – Abs. blank / Abs. control).

Analysis of DNA damage by comet assay

The DNA damage was evaluated by subjecting the lymphocytes for single cell electrophoresis (comet assay) at a concentration of 1×10^5 cells/mL [62, 63] using comet assay kit (R&D Systems, Minniapolis, MN, USA). The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electrical potential, whereas undamaged super coiled DNA remains within the confines of the cell membrane when a DC current is applied. Evaluation of the comet tail shape and migration pattern allows extent and assessment of DNA damage. The assay was carried out by immobilizing the lymphocytes in a bed of low melting agarose on a slide after culturing the lymphocytes for a period of 16 h. The immobilized cells were subjected to gentle lysis using lysis buffer containing 2.5 M NaCl, 0.1M EDTA, 0.01M Tris, 1% sodium-lauryl sarcosinate and 1% Triton X-100. The cells were then subjected to electrophoresis in TBE buffer (0.89 M Tris, 0.88 M boric acid and 0.02 M EDTA; pH 7) in a horizontal electrophoresis apparatus, after treatment with alkali (0.3 M NaOH) to denature the DNA and hydrolyze the sites of damage. The electrophoresis was carried out under potential difference of 1 V/cm for 10 min. The slides were fixed and scored under epi-fluorescence microscope (Olympus, BX100, New York, USA) using fluorescent dye, SYBR green. Depending on the tail length, the cells were categorized into (i) nominal and (ii) long tail by scoring 100 cells. Cells, without tail were scored separately. A positive control was also separately run by treating the cells with 100 μM H_2O_2 for 20 min at 4°C.

DNA laddering analysis for cell death

DNA laddering analysis was carried out by collecting cell pellet (5×10^5 cells) in an Eppendorf tube after 16 h of incubation. Cells were lysed by the addition of 20 μL of lysis buffer (TE buffer containing 0.8% SDS) mixed well, and incubated at 37°C for 120 min with RNAse A/T1 cocktail mix. Proteinase K (10 μL) was added and incubated at 50°C overnight. The samples were loaded into dry wells of 1.5% agarose gel containing 0.5 μg/mL of ethidium bromide after mixing with 5 μL of 6X DNA loading buffer. The samples were subjected to electrophoresis at 35V for 4 h [48]. DNA ladders were finally visualized by UV light and the band intensity was quantitated by charge-coupled device (CCD) based digital image analysis system (UVItec, UVIsoft Image acquisition and analysis software, Cambridge, UK).

Flow Cytometric analysis of apoptosis

Flow cytometric quantitation of apoptotic cells was carried out using Annexin V-Biotin Apoptosis Detection kit (R&D Systems, Minniapolis, MN, USA), which uses Annexin V- Biotin conjugate for detection of cell surface changes that occur early in the apoptotic process. Lymphocytes (1×10^6 cells) were collected by centrifugation at 500 ×g for 5 -10 min at 22°C after 16 h of exposure to magnetic fields. Cells were washed by re-suspending in 500 μL of PBS and pelleted by centrifugation at 500 × g. The cells were incubated in 100 μL Annexin V incubation reagent in the dark for 15 min at 22°C followed by incubation with binding buffer containing streptavidin- Fluorescein isothiocyanate (FITC) conjugate in the dark for 15 min at 22°C. Samples were analyzed by flow cytometry (Becton Dickinson, FACS Calibre, USA).

Spectrofluorometric analysis of caspase 9 activity

The assay of caspase 9 activity was carried out using fluorometric assay kit (R&D Systems, Minniapolis, MN, USA). The lymphocytes that are subjected to magnetic fields for 16 h were first lysed to collect their intracellular contents. The cell lysate was then tested for protease activity by the addition of a caspase - specific peptide (LEHD) that is conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (AFC). The cleavage of the peptide by the caspase releases the fluorochrome that when excited by light at 400 nm wavelength, emits fluorescence at 505 nm. The level of caspase enzymatic activity in the cell lysate is directly

proportional to the fluorescence signal detected by a spectrofluorometer (Hitachi F-4500; Hitachi, Tokyo, Japan). The caspase 9 activity was expressed as fluorescence intensity (FI)/mg protein by estimating the protein content in the cell lysate using detergent friendly bicinchoninic acid method [64].

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